Identification of leukemia and cancerous leukocytes based on biophysical properties

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Introduction

Every three minutes, someone in the United States is diagnosed with blood cancer, the most common of which being Leukemia, or the cancer of white blood cells.[1] Differentiating between healthy and cancerous white blood cells through traditional techniques has proven to be difficult and inconsistent. This is due to the similarities in morphology and appearance between healthy cells and their cancerous counterparts. Currently, the most accurate method for detecting cancerous cells is the labeling with fluorescent antibodies and enumeration using flow cytometry. Flow cytometry can be time consuming, expensive, and prone to errors [see if there are limitations in the antibody markers in terms of specificity or selectivity].

In recent years, there have been rapid advancement in the field of cellular biomechanics. Researchers having using tools, such as Atomic Force Microscopy (AFM), to mechanical characterize and image biological samples. AFM is a probing technique that uses micro cantilevers to apply contact forces to samples. AFM has been used to generate force maps, calculate elasticity, determine relaxation constants, and characterize other mechanical values of various cell types.

In the fall of 2014, a microfluidic chip that sorts cells based on their biophysical properties was developed. This chip initially sorted two cancer cell lines (K562 and HL60) as a proof of concept for the technology. It is the goal of this study, to mechanically characterize healthy blood cells and cancerous cells for application in this technology.[2] The authors believe that cells have unique biophysical properties that can used to identify one cell from another, and, that by using AFM, the properties can be recorded and used for future applications.

The goal of the research described in this thesis is to determine size, elasticity, and viscoelasticity of 4 leukocyte cell types, neutrophils, lymphocytes, HL60, and Jurkat. HL60 is a representative of Acute Myeloid Leukemia (AML), and Jurkat is representative of Acute T cell leukemia (ALL). Neutrophils and

Lymphocytes are two different types of healthy white blood cells. We believe it is possible to differentiate cells only using mechanical characteristics.

Literature Review

Atomic Force Microscopy (AFM) is an extremely powerful tool in the mechanical characterization of cellular and subcellular systems. This probing technique has been applied to a range of physiological and nonphysiological systems with much success. The area of cell biophysical characterization has been explored with the use of AFM, but there are still great strides to be made. Our research aims to characterize multiple mechanical properties of cancerous cells and compare the results to the healthy cellular counterparts.

Past research has shown that physiological systems can be uniquely characterized by the mechanical properties they present. Espino, Shephard, and Hukins conducted a study on bovine knee joint cartilage. The study showed that thicker cartilage is predisposed to failure and that thinner cartilage is less stiff.[3] The study was able to completely characterize a biological system using mechanical studies, which allows for further investigation in degenerative joint diseases. Additionally, there has been a great deal of mechanical studies done on the neural system. Speddin et al. performed a study on various types of mice neurons. They used AFM to determine the stiffness on the neuron, fluorescently labelled the actin to access the cytoskeletal structure, and combined both to determine the state of the neuron's health.[4] This level of information can be used to access the progression of Alzheimer's, the severity of a concussion, and much more. These are just two examples of how mechanical characterization of biological systems can lead to tangible benefits.

In the Espino study, the samples were tested using Dynamic Mechanical Analysis (DMA). DMA is an effective tool in determining the mechanical boundaries on large samples, but cannot be used in a cellular system. That is why AFM is used for microscopic samples.

Mathur et al's research has shown that AFM probing techniques can be used on cardiac and skeletal muscle to determine each muscle type's stiffness. In order to measure the samples, this study used a specific set of protocols that has been repeated a number of times and was repeated in the current study. First, the researchers used an inverted microscope in order to view the tip and sample from below. The researchers then calibrated for a constant spring force by measuring the ambient thermal vibrations. Finally, Mathur's group use a conical tip in contact mode to apply a load to the sample and determine the elastic modulus.[5] These procedures were generally followed by Mulieri's Myocardial Stiffness Study, Spedden's Neuron mechanics study, and much more. [6]

In. Xu et al's study of human neutrophils, which did not use AFM, the team applied the desired load via a pressure on a polystyrene bead. The bead is approximately 2.5 microns in diameter, much smaller than the cell, but can apply a distributed load.[7] This idea has been adapted for use by AFM. By adhering a polystyrene bead to the end of a microcantilever, more distributed load can be applied to a sample. This method was used previously by Sulchek in a study of leukemia cells.[8] Using this technique allows for a stiffness measure that is far more representative of the entire cell. An unbeaded cantilever tip can be biased by the cell physiology, meaning if a measurement is run over the nucleus of a cell, the AFM will read a stiffness that is too high.

Aside from of stiffness (elasticity) there are other properties that can aid in the mechanical characterization of a sample, one of which is viscoelasticity. Darling et al. performed a study on zonal articular chondrocytes. This study used all of the previously stated procedures, including a bead cantilever. To determine a cell's viscoelastic properties, a load was applied to a cell under a constant force and held for a set amount of time. Two time relaxation values are then calculated and used to characterize the cell's viscoelastic properties. In this study, the measurements from AFM were compared to measurements obtained from the more established method of micropipette aspiration in order to determine AFM's accuracy and precision. Darling et al. found that AFM was not statically different from micropipette aspiration, and that it is indeed a good tool for viscoelastic measurements.[9] As pointed out

before, there have been AFM studies on various leukemia and leukocyte cell types in the past. However the research performed has been limited in its scope. While Xu et al. studied the stiffness of neutrophils, they failed to measure the viscoelastic properties of the same samples. Darling et al. took it one step further by calculating both the elastic and viscoelastic properties of the zonal articular chondrocytes. The research that that is currently being proposed will calculate both viscoelastic and elastic properties of cancerous cells and compare them to their healthy cell counterparts. This would allow for a broader understanding on what makes cancerous cells so much more different than their healthy counter parts. Additionally, this knowledge can open further fields of study that combine gene analysis, with AFM measurements, in the hopes of predicting cancer before it even happens.

Methods and Materials

Cancer Cell Culture

All cell lines were received from ATCC lab supplies and cultured as direct. Cell media was derived by mixing Fetal Bovine Serum (FBS) and Dulbecco's Phosphate-Buffered Saline (DPBS) and renewed via centrifugation every 2-3 days for all cell types. The media for K562, Jurkat, and HL60 contained 10%, 10%, and 20% solution, respectively, of FBS and the remaining volume was DPBS.

Blood Cell Isolation

Healthy Leukocytes cannot be cultured. In order to run experiments leukocyte samples needed to be harvested the day of the experiment. First, a donor blood sample needed to be collected. The process of centrifugation and the application of an RBC lysis buffer was used to lyse red blood cells and remove the particles from the healthy leukocytes.

Cell Plating

This study coated the entire surface of fluoro-dishes (check maker) with 10 microliters of Poly-l-Lysine. After 10 minutes, a sample of cells in suspension was pipetted in the flouro-dish and allowed to minutes

to adhere to the surface. After another 10 minutes, the remaining cell media was aspirated off and 3ml of DPBS was pipetted into the flouro-dish. These steps electrochemically adhere cells to the surface of the flouro-dish and prevent them from moving during an AFM measurement.

Atomic Force Microscopy

This study used an Atomic Force Microscope with an inverted microscope produced by Asylum Research to perform force measurements. This system uses an inverted microscope in order to view samples during experiments. Additionally, micro-cantilevers from xyz were used. This study calibrated the spring constant for Tip C on Bruker's microcantilevers by measuring the stiffness of glass in 3ml of DPBS. Flour-dishes were secured to the system by magnets. After calibration, the flouro-dish of DPBS was removed from the system and a dish with cells replaced it.

In order to record Young's Modulus and 2 time relaxation constants, the AFM was set to apply a constant 1 N of force with a 5 second dwell time. Cells were select without discrimination to ensure that the sample population was as random as possible. A minimum of 30 cells were measured for each cell type. Also, images of each cell were taken. The diameter of each cell was calculated using the image processing program ImageJ. Only samples that had viable measurements for all for parameters (cell diameter, Young's Modulus, InvTau1, and invTau2) were used in a sample population.

ROC Analysis

ROC analysis was performed to examine the quality of separation between cell populations with the addition of identifying physical parameters. This analysis generates 4 plots that start with a comparison between the cell populations elastic modulus. This quality of the separation is quantified with an AUC, area under curve, value. A higher AUC is indicative of a higher quality of separation between the two populations, which indicates easier identification. The first subplot only separates the population by elastic modulus. The second subplot separates the populations

by elastic modulus and cell diameter. The third subplot separates the populations by elastic modulus, cell diameter, and τ_1^{-1}. The fourth subplot separates the cell populations by elastic modulus, cell diameter, τ_1^{-1}, and τ_2^{-1}. By accessing the AUC's for each plot, it can be determined if it the addition of identifying parameters improve the identification of cell populations.

Results

This study found significant differences in the elastic values, cell diameters, and τ_{1-} between healthy and cancerous cells (Table 1). Lymphocytes and neutrophils are stiffer than there Jurkat and HL60 (397 Pa and 538 Pa, respectively). Neutrophils have a larger diameter than HL60 (5.24 μm) and Lymphocytes are smaller than Jurkat (1.88 μm), and Lymphocytes have a smaller T_1^{-1} than Jurkat.

Cell Types	*E (Pa)*	τ_1^{-1} *(s^{-1})*	τ_2^{-1} *(s^{-1})*	*Diameter (μm)*	*n*
Neutrophils	*842 ± 526 **	*1.39 ± 1.32*	*4.50 ± 3.61*	*15.99 ± 1.53^{++}*	*25*
HL60	*304 ±184 **	*1.50 ± .65*	*6.91 ± 6.25*	*10.75 ± 1.56^{++}*	*29*
Lymphocytes	*495 ± 210 ***	*1.12 ± .39^{+}*	*8.14 ± 7.55*	*10.37 ± 1.115*$^{+}$*	*26*
Jurkat	*99 ± 46 ***	*1.53 ± 1.55^{+}*	*10.89 ± 19.02*	*12.25 ± 1.53 *$^{+}$*	*25*

Table 1. Biophysical Properties of Healthy Leukocytes and Cancerous Cell types

All samples were probed with an Asylum Research Atomic Force Microscope and mechanical values were calculated in the data analysis tool pack in an Asylum Research Software package. The healthy cells were statistically compared to the cancerous cells using a Student T-Test.

This study also performed an ROC Curve analysis to quantify the separation of each cell type as parameters where added. Figure 1 displays the results when HL60 is separated from Neutrophils using this method. An AUC over .5 indicates that there is a significant amount of separation between

Neutrophils and HL60 cells. The AUC increase from .63393 to .74026 with the addition of cell diameter, but the ROC only slightly increases with the addition of τ_1^{-1} and τ_2^{-1}.

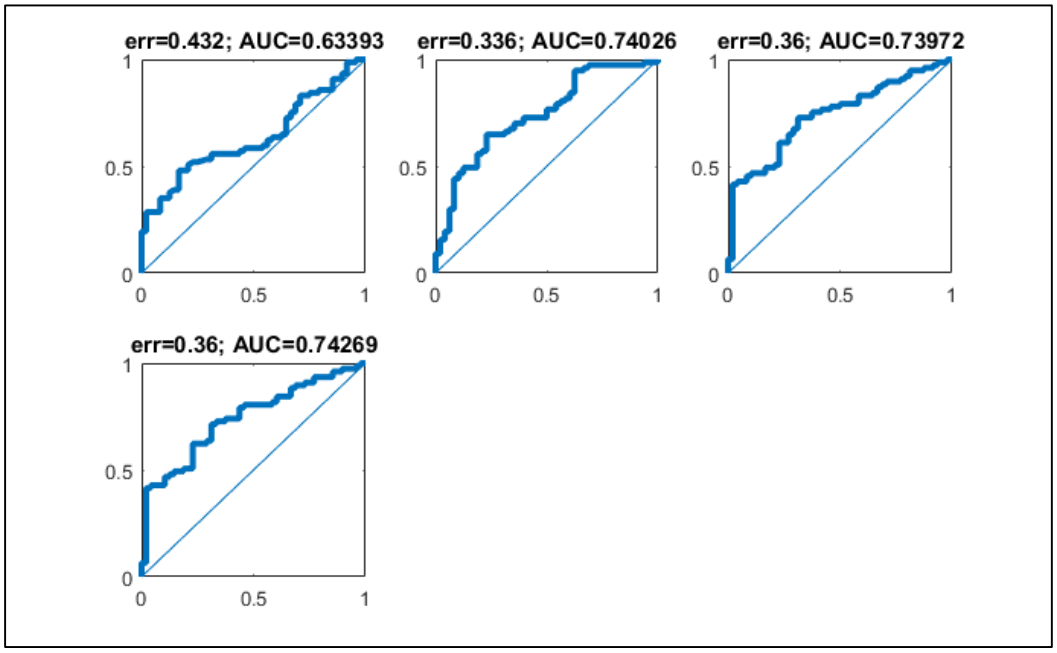

Figure 1. ROC analysis of separation between HL60 and Neutrophils
The quality of separation has an increasing trend with the increase of identifying mechanical properties. The elastic modulus and cell diameter are the larger driving factors for separation between HL60 and neutrophils. τ_1^{-1} and τ_2^{-1} are weaker driving factors, but these properties do have positive impact

Figure 2 displays the results of ROC analysis when comparing the Jurkat cells to Lymphocytes. Comparing the populations elastic modulus results in an AUC of .97812. Adding the other 3 parameters results in an increase of .0179 in the AUC of the anaylsis.

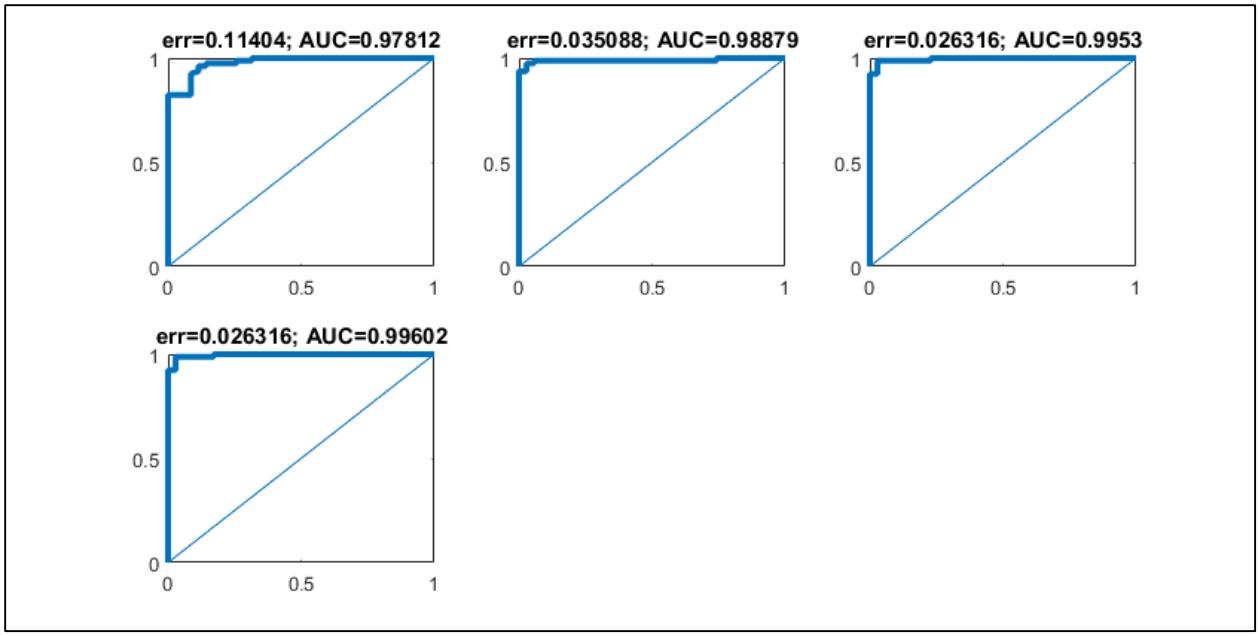

Figure 2. ROC analysis of separation between Jurkat and Lymphocytes

Elastic modulus is the largest driving force when separating Jurkat and lymphocytes. It almost results in a perfect separation with an AUC of .97812. Adding the other parameters only slightly increases the AUC of the curves, but that is due to the fact that elastic modulus was such a good identifier by itself.

Conclusion

This study has shown that there are distinct measurable differences in the mechanical properties of healthy and cancerous blood cells. There are significant differences in all of the Elastic Moduli and Cell Diameters. These findings were strengthen with the use of ROC curve analysis. The ROC analysis showed that there is clear distinction between the cell types when identifying the populations based of mechanical parameters. Again, ROC showed the elastic modulus and cell diameter are the 2 main drivers of cell separation between the sample populations. It has been a long held understanding that cancer cells have difference mechanical properties. This results are consistent with previous studies have shown that populations of cells can be separated based on these specific biophysical properties through the use of a

microfluidic chip.[2,8] This type of technology has the potential to offer a simple, routine blood test as an alternative to a bone marrow biopsy as a diagnostic procedure.

Discussion and Future Works

This study has accomplished its original goal by demonstrating that leukemia cells of cancerous phenotypes possess significantly different mechanic properties than healthy phenotypes. The methods of this study can be built upon to develop a deeper understanding of cancer cell mechanics. There are many different cellular components that are factored into a cell's mechanics. Fluorescently tagging various cytoskeletal structures and organelles would lead to a more detailed understanding of a cell's inner workings. Additionally, this study can be expanded by repeating its methods on a range of cell types. This would lead to a better understanding of cancers as a whole.

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